PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	**Screening for Congenital Cytomegalovirus Infection Using Newborn Urine Samples Collected on Filter Paper: Feasibility and Outcomes from a Multi-centre Large-scale Study**
AUTHORS	Koyano, Shin; Inoue, Naoki; Oka, Akira; Moriuchi, Hiroyuki; Asano, Kimisato;Ito, Yushi; Yamada, Hideto; Yoshikawa, Tetsushi; Suzutani, Tatsuo; Fujieda, Kenji

VERSION 1 - REVIEW

REVIEWER	***Jutte J.C. de Vries, MD*** Dpt. Medical Microbiology Leiden University Medical Center (LUMC) The Netherlands
REVIEW RETURNED	24-Mar-2011

THE STUDY	OVERALL: This is a very interesting study, exploring the major topic of potential sceening newborns for congenital CMV infection. It is a large study, with an essential goal, and important data are collected. The major points mentioned below should be adequately addressed. MAJOR REMARKS -Since the goal of the study was to test feasibility, please accentuate logistic and practical feasibility more throughout the paper (results, discussion, and abstract). Since predictive values are crucial when using large-scale screening tests, please calculate and describe positive and negative predictive values in the abstract. In line with that, throughout the whole paper, please provide exact numbers concerning the logistic feasibility: e.g. how many filter papers were e.g. not taken (compared to the total no of births at all sites), were there cards that were not posted, missing? Wat was the mean age (and range) at which confirmatory liquid urine samples were collected for virus culture? (<3 wks?) And, since only hospitalized newborns were included (how many of the labours do take place at home in Japan), will this be feasable in other countries with more labours performed at home? And, was there any trouble with automated punching, etc. Please address the logistic feasability aspect more. -Please make sure that it is clear for the reader that the sensitivity no's given do not address comparison with the gold standard (since the urine-filter negative samples are not checked by means of urine culture). -Please mention exact numbers in the figure, to clarify the methodology. (see below) It can be presented more clear and more precise. -It is crucial that a native English speaker checks the grammar. IN MORE DETAIL:

	Title: delete large-scale. multi-centre is sufficient Abstract: objectives: replace "that allows"for "for" Abstract: results: after "most of the cases", mention the number identical versus the number of total strains tested (21/25). IMPORTANT Abstract: results: replace “The DBS-based assay had a 75% sensitivity of our urine-based assay” by “Compared to the filter-based urine samples, the DBS assay had a sensitivity of 75% (x/xx).” Please mention numbers x. Abstract IMPORTANT Since predictive values are crucial when using large-scale screening tests, please calculate and describe positive and negative predictive values in the abstract! Introduction: Replace "because many cases have late-onset, standard newborn hearing" for "because many cases have late-onset SNHL", early newborn hearing screening" Introduction: Delete the whole sentence "It is also crucial ... developed." Introduction: in line with ref nr 9 and 10, replace ïnsufficienlty sensitivity"for "of limited sensitivity". Introduction: delete the sentence "if DNA purification, rather than, ... currently estimated", or add a reference to the statement in which the estimation is published. Introduction: last line: replace "diseases in Japan, ... infection" for "disease, and to identify .... infection in Japan." Methods: line 4: replace "pieces" for "piece" methods line 7: mention that this PCR amplifies which fragment of CMV. methods line 7: replace 1.11 days for 1.1 or 1 day, etc for 1.22 days line 12: either delete "at the same time or within a short period", or, mention the specific period in days IMPORTANT Mention the time period within the confirmatory liquid urine sample was collected after birth (crucial: was it within 3 weeks), mention mean and range (minimum-maximum). methods, second paragraph: replace "almost all"for the actual percentage of newborns. IMPORTANT Methods: mention whether or not an internal control PCR, to control for inhibition was used. Methods, paragraph clinical and audiol..: Delete sentence "although interpretation of mild .... imaging". Reconfirm (in the methods) or clarify that the hospitals that tested IgM, also screened all neonates using urine filter paper. IMPORTANT Figure: mention exact number (or %) of patients with informed consent (and refusal) Mention in figure how many IgM tests were performed, and how many MRI/CT s were performed Results: mention how many samples were discrepant in the 1st vs 2nd PCR. Delete sentence "causes of 4 false positive .. are not clear". Results, line 6, detele “Although”, start with “Five.. etc. Results line 9: Clarify "all 50", since there were 70 samples.

	results, paragraph "characteristics": replace "chrorioretinitis"for "chorioretinitis" results: clarify "brain imaging was not available for some cases", since 4 lines above, it seemed that all 66 cases underwent brain imaging. Or change the /66 written above. Mention whether the 6 treated newborns had symptoms. results: mention the area in which the nurse was active (child care?). results, technical issues: Delete the sentence "there are a couple... issues. First" and start with "CMV-specific.. etc" IMPORTANT Mention, after the sentence "only 75%....", that an accurate comparison with the current gold standard, virus culture, cannot be made with the study method used. The authors mention themselves that their earlier data suggested a sensitivity of 90% of their DBS assay. Results: last paragraph; delete "but only at a few study sites and delete"such adverse events are avoidable" IMPORTANT the conclusion that screening using filter paper in diapers is feasable can only be drawn with more appropriate numbers concerning the logistic feasability: e.g. how many filter papers were missing, or late (so that urine sampling to confirm the diagnosis was later than 3 wks), etc. And, since only hospitalized newborns were included (how many of the labours do take place at home in Japan), will this be feasable in other countries with more labours performed at home? And, was there any trouble with automated punching, etc. Please address the logistic feasability aspect more. Discussion delete "suggesting a greater role of primary infection in congentital .. infection" Delete (since it is too speculative): "although the major transmission ..... hygiene practices". Rewrite the senctence "the large volumes or urine produced daily ... will make it difficult"too speculative. IMPORTANT Discussion: Since predictive values are crucial when using large-scale screening tests, please calculate and describe positive and negative predictive values! table 1: describe in legend what type 1 and 2 sites are (such as in methods) tabl 2 : one or more siblings per case? table 3: delete column IK and study area (non informative for reader Figure 2 Please add to the vertical axis : (blood/ liquid urine)

REVIEWER	***Klaus Hamprecht, MD, PhD*** University Hospital of Tuebingen Institute of Medical Virology

	Elfriede Aulhorn Str 6 72076 Tübingen Germany There are no competing interests
REVIEW RETURNED	22-Apr-2011

THE STUDY	I agree with the presented documents of the manuscript.
GENERAL COMMENTS	To the authors Koyano et al., present a large epidemiological study on the prevalence of congenital CMV (cCMV) infection in Japan. In this paper included are some interesting side aspects like examination of siblings of infected infants and genotyping of shedded viral strains. The authors present a well designed study using an interesting new approach for newborn screening with clear results and impact for the epidemiology of congenital CMV. However there are some-mostly minor- draw-backs, which may be optimized for the interested readership: 1.) Introduction, second section, line 48, and 53 (minor point) The urgent need of an alternative assay to DBS screening as argued with references 9, 10, and 13 is also underlined by Göhring et al, 2010. 2.) Methods; Study design/Confirmation of infected cCMV cases (minor point) Since the authors found 4 false positive cases out of 70 cCMV cases (nearly 6% of all), the intrinsic controls for confirmation of a putative cCMV-infected infant are very important. The authors should point out in discussion, how many time was lost for recollection of urine, that means-did all parents really immediately revisit the hospital? In how many cases the second urine sample could be taken after 10 days post partum? Its known that PCR data from postnatally via breast milk transmitted virus may interfere with congenitally transmission, if the materials are collected after two weeks post partum. 3.) Methods; qPCR screening (major point) Since there is a clear correlation of sensitivity of detection to the input DNA/area of filter spots, the authors should also discuss their approach using only 1x 3mm filter spot for qPCR screening (page 6, line 58) while 4x 3mm spots were used (page 7, line 6) for confirmation tests. Interestingly, the authors omitted any DNA extraction step for routine screening of heir filter spots. Did the authors use an internal PCR control? It would be helpful, if the authors could show primary data (ct values) of artificially spiked Whatman filters wether the presence of urine components does not interfere with the amplification sensitivity. 4.) Methods; Lack of transparence regarding type-1/type-2 center (page 6, line 46), (minor point) Since the epidemiological results strongly differ between type-1 and so-called type-2 sites (Table 1), the authors should point out which of the hospitals cited in the acknowledgement belong to type 1 or 2, respectively. 5.) Methods; Study design incongruity (page 8, line 3-8), (major

	point) Two study centers obviously performed maternal CMV serology. Why not all? This would have been very helpful for the epidemiological results. It is neither given when (at birth? Or during pregnancy?) blood for serology was taken. And the most important point: which test system was used for serology? 6.) Results; qPCR validity, page 9, line 22 (major point) “5 of 70 positive cases were weakly positive”in initial screening. Is the detection of 300 000 copies of CMV DNA (page 9, line 23) correctly named “weakly”? For validity of all presented data the authors should try to find an explanation for the false positive DNA findings in 4 out of 70 cases? Screenin a large population as done in theis study, this may influence the results. 7.) Results, page 9, line 41-53, Table 1 (major point) There is no satisfying explanation for the huge difference between “typical manifestations at birth” with 8.6% in type-1 sites versus 38.7 % in type-2 sites? Its important to clarify that, otherwise remains something like a bias in the study design. It should be outlined in detail: What are “typical clinical manifestations at birth”? The definition is not given in MM. What is the study design rationale for antiviral treatment after birth? Was treatment only restricted to type-2 sites? Was there any criteria which infant has to visit type-1 or type-2 sites? 8.) Results, page 10, line 3 (major point) The authors present with 22.7% symptomatically infected infants a very high incidence of severe cCMV. In almost all other studies only about 10% of all infected infants (in this study 6.6 infants) are found. 9.) Results, Table 1 (minor point) The authors should give the exact number of infants and not only percent data (line 11-15) 10.) Results, page 10, line 15-22 (major point) Please give detailed definitions what do you define as “symptomatic”. What is the reason that type -2 centers did nearly find 5-fold (38.7%) as much as type-1 hospitals (8.6%) “typical clinical manifestations” at birth? 11.) Results, page 10, line 32-39 (minor point) It would be very interesting, to get information on the 6 cases of cCMV with GCV treatment. 12.) Results, page 11, line 46-60 (minor point) Could the authors please provide the test they used for IgM testing. 13.) Results, Missing data outlined in “Methods” (minor point) In Methods (page 6, line 29-36) there is outlined a more detailed virological analysis of the identified cCMV cases. Unfortunately these data are missing in “Results”. Therefore the authors should cancel these announcements in Methods. Why only in 12/66 infected infants DBS versus urine was analysed with qPCR? Dear authors please feel not disappointed, these comments are intended in a really constructive manner.

VERSION 1 – AUTHOR RESPONSE

MAJOR REMARKS

1) Since the goal of the study was to test feasibility, please accentuate logistic and practical feasibility more throughout the paper (results, discussion, and abstract). Since predictive values are crucial when using large-scale screening tests, please calculate and describe positive and negative predictive values in the abstract. In line with that, throughout the whole paper, please provide exact numbers concerning the logistic feasibility: e.g. how many filter papers were e.g. not taken (compared to the total no of births at all sites), were there cards that were not posted, missing? What was the mean age (and range) at which confirmatory liquid urine samples were collected for virus culture? (<3 wks?) And, since only hospitalized newborns were included (how many of the labours do take place at home in Japan), will this be feasible in other countries with more labours performed at home? And, was there any trouble with automated punching, etc. Please address the logistic feasability aspect more.

> We modified the whole manuscript as far as possible to clarify the logistic and practical feasibility. The details are described below in response to the reviewer's precise comments (items 3, 10, 11, 15, 16, 20, 26).

2) Please make sure that it is clear for the reader that the sensitivity no's given do not address comparison with the gold standard (since the urine-filter negative samples are not checked by means of urine culture).

> In this revised manuscript, we described the lack of comparison with the gold standard as one of the limitations of this study, both in the Article Summary and in the Discussion section.

3) Please mention exact numbers in the figure, to clarify the methodology. (see below) It can be presented more clear and more precise.

> As described below, we modified Figure 1.

4) It is crucial that a native English speaker checks the grammar.

> We sent this revised manuscript to a professional English editing service before submission. Based on their suggestions, we corrected the text extensively without changing the contents.

IN MORE DETAIL:

5) Title: delete large-scale. multi-centre is sufficient
Abstract: objectives: replace "that allows"for "for"
Abstract: results: after "most of the cases", mention the number identical versus the number of total strains tested (21/25).

> We modified the title and abstract as suggested.

6) Abstract: results: replace "The DBS-based assay had a 75% sensitivity of our urine-based assay" by "Compared to the filter-based urine samples, the DBS assay had a sensitivity of 75% (x/xx)." Please mention numbers x.

> Since we realize that "a 75% sensitivity" is a confusing and inadequately strong wording, we modified the sentence as follows: "CMV DNA was undetectable in 3 out of 12 retrievable DBS specimens."

7) Abstract: Since predictive values are crucial when using large-scale screening tests, please

calculate and describe positive and negative predictive values in the abstract!

>Although we understand that predictive values are crucial when using large-scale screening tests, our study had a limitation in counting false-negative cases. Therefore, in the abstract and discussion sections, we stated the positive predictive value and our limitation in counting false-negatives.

8) Introduction: Replace "because many cases have late-onset, standard newborn hearing" for "because many cases have late-onset SNHL", early newborn hearing screening"
Introduction: Delete the whole sentence "It is also crucial ... developed."
Introduction: in line with ref nr 9 and 10, replace ïnsufficienlty sensitivity"for "of limited sensitivity".
Introduction: delete the sentence "if DNA purification, rather than, ... currently estimated", or add a reference to the statement in which the estimation is published.
Introduction: last line: replace "diseases in Japan, ... infection" for "disease, and to identify .... infection in Japan."
Methods: line 4: replace "pieces" for "piece"
methods line 7: replace 1.11 days for 1.1 or 1 day, etc for 1.22 days
line 12: either delete "at the same time or within a short period", or, mention the specific period in days

> We accepted all of those editorial suggestions and modified the text.

9) methods line 7: mention that this PCR amplifies which fragment of CMV.

> We clarified it in the next “qPCR for screening” section.

10) Mention the time period within the confirmatory liquid urine sample was collected after birth (crucial: was it within 3 weeks), mention mean and range (minimum-maximum).

> Specimens for confirmation were collected at the age of 15.8 ± 3.8 days (range 7-21 days). We added these numbers in the text and in Figure 1B.

11) methods, second paragraph: replace "almost all"for the actual percentage of newborns.

> We described the proportion of babies recruited for enrollment (>99.9% of all born at the study sites) and refusal (1.3%) in the text and Figure 1B.

12) Methods: mention whether or not an internal control PCR, to control for inhibition was used.

> We added the following sentences to the text. “Although it is ideal to include an internal control to assure the absence of PCR inhibition, we did not do so in this study for the following reasons: 1) although we initially spiked irrelevant DNA/primers/probe into the qPCR reaction mixture for screening (n=200 urine-filters) to check the efficiency of qPCR, no cases of inhibition were observed, 2) our previous study demonstrated a close correlation in viral load measurements between the urine-filter-based qPCR assay and the common qPCR assay using DNA samples purified from liquid urine, and 3) to reduce costs as far as possible.”

13) Methods, paragraph clinical and audiol..: Delete sentence "although interpretation of mild .... imaging".

> We deleted the above as suggested.

14) Reconfirm (in the methods) or clarify that the hospitals that tested IgM, also screened all neonates

using urine filter paper.

> We added the following sentence to the text: “All of their babies except for refusals were enrolled for CMV screening after birth”

15) Figure: mention exact number (or %) of patients with informed consent (and refusal)
mention in figure how many IgM tests were performed, and how many MRI/CT s were performed

> We added the information into Figure 1B.

16) Results: mention how many samples were discrepant in the 1st vs 2nd PCR.

> All 1st PCR positives were positive in the 2nd PCR using DNA eluted from the original filters. We modified the text for clarification as follows, “and all of them were confirmed positive by the second PCR using DNA samples recovered from the urine-filters.”

17) Delete sentence "causes of 4 false positive .. are not clear".
Results, line 6, detele “Although”, start with “Five.. etc.

> We modified the text as suggested.

18) Results line 9: Clarify "all 50", since there were 70 samples.

> We modified the text to clarify that virus isolation was perform for 50 samples.

19) results, paragraph "characteristics": replace "chrorioretinitis"for "chorioretinitis"

> We corrected the typo.

20) results: clarify "brain imaging was not available for some cases", since 4 lines above, it seemed that all 66 cases underwent brain imaging. Or change the /66 written above.

> We modified the text to clarify that brain images were available for 58 cases (p.12).

21) Mention whether the 6 treated newborns had symptoms.

> We modified the text to clarify their clinical symptoms and abnormalities (p.12).

22) results: mention the area in which the nurse was active (child care?).
> We described that she worked in the department of internal medicine.

23) results, technical issues: Delete the sentence "there are a couple... issues. First" and start with "CMV-specific.. etc"

> We deleted the above as suggested.

24) Mention, after the sentence "only 75%....", that an accurate comparison with the current gold standard, virus culture, cannot be made with the study method used. The authors mention themselves that their earlier data suggested a sensitivity of 90% of their DBS assay.

> We assume that reviewer referred to “a sensitivity of 90%” from the sentence in the Materials and Method section describing “The efficiency of DNA recovery from DBS was >90%.” as well as from the

sentence in our previous publication (Inoue and Koyano, 2008) describing “The recovery efficiencies of method B from 1 blood specimen containing 42.9 copies per 3 ul and the other blood specimen containing 1094.7 copies per 3 ul were 91.4% and 93.9%, respectively.” Since the numbers were obtained from filters experimentally spiked with two blood specimens, they don’t indicate the sensitivity of the screening which is the result of a combination of the assay sensitivity and the viral loads in the specimens. If viral loads in some of the blood specimens in the screened population are very low; for example, less than 10 copies per 3 ul, the “sensitivity” of the screening itself might become smaller than the “sensitivity” of the assay used. In our context, the important point is not the sensitivity of the assay itself, but the low viral loads in the blood specimens.
Because we think that our improper usage of the word “sensitivity” both in the abstract (a 75% of sensitivity) and in this paragraph (low sensitivity of DBS-based screening) confused the reviewer, we re-phrased the text to clarify the point as follows: “Viral load estimates from the DBS specimens from the cases (n=12) also showed fair agreement with those determined using blood specimens. Importantly, the total CMV DNA load in 3-4 discs of DBS specimens was far less than that in a single urine-filter disc, and 3 out of 12 DBS specimens were negative in the PCR assay, the sensitivity of which was previously demonstrated to be >90%.”

25) Results: last paragraph; delete "but only at a few study sites and delete"such adverse events are avoidable"

> We deleted the phrase as suggested.

26) the conclusion that screening using filter paper in diapers is feasible can only be drawn with more appropriate numbers concerning the logistic feasibility: e.g. how many filter papers were missing, or late (so that urine sampling to confirm the diagnosis was later than 3 wks), etc. And, since only hospitalized newborns were included (how many of the labours do take place at home in Japan), will this be feasible in other countries with more labours performed at home? And, was there any trouble with automated punching, etc. Please address the logistic feasability aspect more.

> We added the following logistic information into the text.
a) Numbers of filters provided and their return rate (p.6)
b) Main reasons for the non-return of filters (p.6)
c) All filters were accounted for, as NIID sent each study site an EXCEL file containing information regarding filter ID, date of receipt of the specimens, and test results after each qPCR run, and each study site added the results to the newborns’ medical records. There were about 20 occasions on which the study sites e-mailed or called NIID because of typographical error in the filter ID in the file (p.6).
d) Numbers of newborns recruited, refusals, and exclusions (p.7)
e) Cases in which the confirmation was performed after 3 weeks were already explained in the original manuscript (p.11).
f) Almost all babies in Japan are delivered at clinics or at hospitals and stay usually for 5-6 days (p.15).
g) In one of our studies, we provided 30 mothers with filters and an illustration showing how to collect urine from their 1~2 year-old children at home, and found that most mothers were able to send the urine-filters back without any trouble (p.15).
h) Use of an auto-puncher and a bar-code system may increase the assay-throughput and reliability, although we did not try this due to the limited resources (p.15).

23) Discussion: delete "suggesting a greater role of primary infection in congentital .. infection" delete (since it is too speculative): "although the major transmission ..... hygiene practices".

> We deleted the above as suggested.

24) Rewrite the senctence "the large volumes or urine produced daily ... will make it difficult" too speculative.

> We modified the text as follows: " A significant proportion of pregnant mothers may be constantly exposed to CMV at home since, as described above, at least a quarter of young children excrete 0.5-1 L of urine containing >10,000 copies/ml of CMV daily, often for >2 years." (p.18)

25) Discussion: Since predictive values are crucial when using large-scale screening tests, please calculate and describe positive and negative predictive values!

> As described for the item 7), we described the PPV and limitations of our study.

26) table 1: describe in legend what type 1 and 2 sites are (such as in methods)

> We added the description to a footnote for Table 1.

27) table 2 : one or more siblings per case?

> Among the 42 cases with siblings, 34 had one sibling, 7 had two, and 1 had three. We modified the text (p.13) instead of Table 2 to indicate these numbers.

28) table 3: delete column ID and study area (not informative for reader)

> We deleted the above as suggested.

29) Figure 2: Please add to the vertical axis : (blood/ liquid urine)

> We modified the vertical axis label in Figure 2 as suggested.

1) Introduction, second section, line 48, and 53: The urgent need of an alternative assay to DBS screening as argued with references 9, 10, and 13 is also underlined by Göhring et al, 2010.

> Sorry for this oversight. We added this citation (ref.14 in this revised manuscript).

2) Methods; Study design/Confirmation of infected cCMV cases: Since the authors found 4 false positive cases out of 70 cCMV cases (nearly 6% of all), the intrinsic controls for confirmation of a putative cCMV-infected infant are very important. The authors should point out in discussion, how many time was lost for recollection of urine, that means-did all parents really immediately revisit the hospital? In how many cases the second urine sample could be taken after 10 days post partum? Its known that PCR data from postnatally via breast milk transmitted virus may interfere with congenitally transmission, if the materials are collected after two weeks post partum.

> Specimens for confirmation were collected at the age of 15.8 ± 3.8 days (range 7-21 days). We added the number in the method section.
> We are aware of the publications, including the reviewer's excellent works, describing that postnatal transmission of CMV via breast milk causes serious problems in preterm immature newborns. However, there are a couple of reasons that we did not consider the interference from breast milk feeding, if we can collect urine specimens within 3 weeks after birth or obtain dried umbilical cord specimens: 1) urine specimens for the initial screening were collected within 5 days after birth, 2)

virus loads in urine/saliva specimens obtained from congenital cases are usually >106 copies/ml, which is definitely larger than those from postnatally-infected healthy children (104-106 copies/ml at 4 wk), 3) virus isolation from urine was done for most cases, and 4) we can use dried umbilical cord specimens to further confirm congenital infection, if we cannot collect liquid urine specimens in a timely manner or in cases of low viral load in the second urine specimens. We added sentences to discuss the issue (p.16) and a sentence to describe that dried umbilical cord specimens from the cases (n=23) were used for further confirmed (p.11).

3) Methods; qPCR screening (major point) : Since there is a clear correlation of sensitivity of detection to the input DNA/area of filter spots, the authors should also discuss their approach using only 1x 3mm filter spot for qPCR screening (page 6, line 58) while 4x 3mm spots were used (page 7, line 6) for confirmation tests. Interestingly, the authors omitted any DNA extraction step for routine screening of their filter spots. Did the authors use an internal PCR control? It would be helpful, if the authors could show primary data (ct values) of artificially spiked Whatman filters whether the presence of urine components does not interfere with the amplification sensitivity.

> In the previous study (Nozawa et al, 2007), we determined that the detection efficiency by filter-containing PCR was 15-20% and that by PCR using eluates from filter discs was 60%. The data requested by the reviewer were provided in that publication.
> Since only one filter disc can be submerged into 50ul of reaction mixture in a well of a 96-well plate after a short centrifugation, we used only one filter disc for the screening PCR. Simple mathematical consideration of the detection efficiencies (15-20% in qPCR containing urine-filter vs. 60% in qPCR using eluted DNA samples) and the number of used filter discs indicates that both qPCR assays detect almost equivalent amounts of CMV DNA. We added the information to the text (p.8).

4) Methods; Lack of transparence regarding type-1/type-2 center (page 6, line 46): Since the epidemiological results strongly differ between type-1 and so-called type-2 sites (Table 1), the authors should point out which of the hospitals cited in the acknowledgement belong to type 1 or 2, respectively.

> We described the classified study sites in the Methods section (p.7).

5) Methods; Study design incongruity (page 8, line 3-8), (major point) :Two study centers obviously performed maternal CMV serology. Why not all? This would have been very helpful for the epidemiological results. It is neither given when (at birth? Or during pregnancy?) blood for serology was taken. And the most important point: which test system was used for serology?

> In contrast to European countries, only 10% of obstetric clinics and hospitals in Japan conduct routine CMV serology for pregnant women (usually done at 10-20 wk of pregnancy), although we are advocating its importance. Since we did not have enough funding to support serological tests, serological data were available only from the study sites where they conducted tests prior to this study. Three study sites conducted CMV-IgG EIA at 10-20 wk of pregnancy. One of them did also CMV-IgG avidity test at the same time, another site conducted an additional CMV-IgG EIA at 35-36 wk on women seronegative at 12 wk, and the last study site undertook CMV-IgM EIA twice, during the middle and late terms, on seronegative women. In addition, we obtained blood specimens from mothers of the congenital cases after birth (n=31), and these materials were used for gH-based strain-specific serology to examine reinfection with a different strain. We modified the “Strain analysis and serology” in the Method section intensively (p.10).

6) Results; qPCR validity, page 9, line 22 (major point) :“5 of 70 positive cases were weakly positive” in initial screening. Is the detection of 300 000 copies of CMV DNA (page 9, line 23) correctly named “weakly”? For validity of all presented data the authors should try to find an explanation for the false

positive DNA findings in 4 out of 70 cases? Screening a large population as done in this study, this may influence the results.

> We modified expression to clarify that these copy numbers are less than those of most congenital cases (p.11).
> We knew the following: 1) At least one of 4 filters was collected at the same site on the same day when a urine-filter of a confirmed congenital case was collected. Therefore, the filter could have been contaminated. 2) The other three were collected at different hospitals and at different times, but in the same area. All four study sites in this area sent filters to one supervisor who is an obstetrician, and he then sent the filters to NIID. We described the first case in the Discussion section (p.16).

7) Results, page 9, line 41-53, Table 1 (major point) : There is no satisfying explanation for the huge difference between “typical manifestations at birth” with 8.6% in type-1 sites versus 38.7 % in type-2 sites? Its important to clarify that, otherwise remains something like a bias in the study design. It should be outlined in detail: What are “typical clinical manifestations at birth”? The definition is not given in MM. What is the study design rationale for antiviral treatment after birth? Was treatment only restricted to type-2 sites? Was there any criteria which infant has to visit type-1 or type-2 sites?

> Since we expected this difference, we presented our data separately for the type-1 and type-2 sites in the original manuscript. We added descriptions of the characteristics of the type-2 sites in the Method section (p.7).
The presence of a NICU, well-equipped facilities and specialists at the type-2 sites and the referral-based move to type-2 hospitals from type-1 sites can result in an increase in pregnant women with risk factors, such as IUGR, at type-2 sites. As described in the result section, there was a difference between the type-1 and type-2 sites in the proportion of newborns with a birth weight <2,500g (5.1% vs. 13.5%) but not in that of newborns small for GA (5.1% vs. 5.9%). Although ideally we should know the proportions of babies born at type-1 and type-2 clinics/hospitals in our whole country, such statistics are not available. Therefore, the true prevalence of CMV infection and disease in Japan is somewhere between those of the type-1 and type-2 sites. We added descriptions of this issue along with higher incidence of symptomatic cases in the Discussion section (p.16-17).
> We added the definition of “typical clinical manifestations” in the Method section (p.9).
> Prior to initiation of the screening, our Study Group prepared a tentative guideline for antiviral treatment based on the same protocol and inclusion/exclusion criteria that were established in the published clinical trial (Kimberlin et al., 2003). Since our study was not for a clinical trial, each patient was treated based on the tentative guideline, informed consent from the parents of the patients, and IRB approval from the facility where the patient was hospitalized (p.9).

8) Results, page 10, line 3 (major point) :The authors present with 22.7% symptomatically infected infants a very high incidence of severe cCMV. In almost all other studies only about 10% of all infected infants (in this study 6.6 infants) are found.

> We need to note that “symptomatic” does not mean “severe”. For example, cases with SNHL without any other symptoms have no life-threatening “severe” manifestations, but we define those cases as being “symptomatic”. We clarified the definitions (p.9) and added descriptions of this issue in the Discussion section (p.16).

9) Results, Table 1: The authors should give the exact number of infants and not only percent data (line 11-15)

> We modified Table 1 to indicate the actual numbers, and indicated necessary numbers in the text.

10) Results, page 10, line 15-22 (major point) :Please give detailed definitions what do you define as

"symptomatic". What is the reason that type -2 centers did nearly find 5-fold (38.7%) as much as type-1 hospitals (8.6%) "typical clinical manifestations" at birth?

> As described for items 7) and 8), we added definitions in the Method section.

11) Results, page 10, line 32-39 : It would be very interesting, to get information on the 6 cases of cCMV with GCV treatment.

> We added the following information to the text (p.12): "…, 6 newborns, who exhibited clinically obvious symptoms (such as IUGR, petechiae, hepatosplenomegaly, and jaundice) with the following abnormalities, SNHL (n=4), chorioretinitis (n=1), and abnormal brain images (n=3), were treated with …." We will publish further details of the treatment elsewhere in the future.

12) Results, page 11, line 46-60: Could the authors please provide the test they used for IgM testing.

> We described the IgM test used in the Method section (p.10).

13) Results, Missing data outlined in "Methods": In Methods (page 6, line 29-36) there is outlined a more detailed virological analysis of the identified cCMV cases. Unfortunately these data are missing in "Results". Therefore the authors should cancel these announcements in Methods. Why only in 12/66 infected infants DBS versus urine was analysed with qPCR?

> We performed virus isolation, virus load measurements, viral genotyping, and gH-based strain-specific serology as virological analyses. We did not show the comparison of viral loads between symptomatic and asymptomatic cases in the original manuscript due to the limitations in the number of Figures/Tables. In this revised manuscript, we would like to show the comparison as a supplementary figure as a proof, if the editor will allow us to do so.
> Since DBS cards were taken solely for identification genetic metabolic disorders in newborns, we needed to obtain informed consent separately from that for the study on congenital CMV infection. Also we needed to ask the incorporated foundations dealing with genetic metabolic disorders to find the particular DBS cards. These procedures resulted in a limited number of DBS specimens available for analysis. In the revised manuscript, we added the following sentences: "We obtained separate informed consent to use DBS specimens from the cases retrospectively for our study, and requested incorporated foundations handling the DBS specimens. DBS specimens were retrieved from 12 of the cases, eleven of which were asymptomatic and 1 had SNHL at birth."(p.7)

VERSION 2 - REVIEW

REVIEWER	***Jutte J.C. de Vries***
REVIEW RETURNED	03-Jun-2011

GENERAL COMMENTS	All my previous remarks are adequately addressed. Just one minor revision requested: please add in figure 1 B the 70% distributed filter cards that were recovered for screening.

REVIEWER	***Klaus Hamprecht***
REVIEW RETURNED	23-Jun-2011

GENERAL COMMENTS	To the authors: After critical reading the revision I have the impression, that all

	questions arising the first time are now sufficiently corrected or changed. There are only 3 minor points, which should be changed: 1.) Abstract, Results: 30% had clinical manifestations; Discussion, page 16, line 37: typ. clin manifest. were observed in > 20% 2.) Article summary, limitations, line 57: studies on mothers are necessary to be combined... 3.) Please substitute ref 22 (Neuberger) by 22 Hamprecht et al., Lancet 2001. The reason is that in the Lancet paper we present in Table 2 exactly the first PCR detection in postnatal transmission on day 12!! I want to congratulate the author team to their important contribution!

VERSION 2 – AUTHOR RESPONSE

Thank you very much for your review on our revised manuscript. We have revised our manuscript as the reviewers suggested.

Please add in figure 1 B the 70% distributed filter cards that were recovered for screening.
> We added the sentence “70% of distributed filters were recovered for screening” into Figure 1B as suggested.

1) Abstract, Results: 30% had clinical manifestations; Discussion, page 16, line 37: typ. clin manifest. were observed in > 20%
> We modified the description in the Discussion section (page 16) to unify the expression as follows. "...typical clinical manifestations and/or the brain image abnormalities were observed in 30% of the cases."

2) Article summary, limitations, line 57: studies on mothers are necessary to be combined...
> We modified the article summary exactly as suggested.

3) Please substitute ref 22 (Neuberger) by 22 Hamprecht et al., Lancet 2001.
> We replaced the citation as suggested.